

# Single molecule force spectroscopy studies of DNA denaturation by T4 gene 32 protein

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**Abstract.** Single molecule force spectroscopy is an emerging technique that can be used to measure the biophysical properties of single macromolecules such as nucleic acids and proteins. In particular, single DNA molecule stretching experiments are used to measure the elastic properties of these molecules and to induce structural transitions. We have demonstrated that double-stranded DNA molecules undergo a force-induced melting transition at high forces. Force–extension measurements of single DNA molecules using optical tweezers allow us to measure the stability of DNA under a variety of solution conditions and in the presence of DNA binding proteins. Here we review the evidence of DNA melting in these experiments and discuss the example of DNA force-induced melting in the presence of the single-stranded DNA binding protein T4 gene 32. We show that this force spectroscopy technique is a useful probe of DNA–protein interactions, which allows us to obtain binding rates and binding free energies for these interactions.

## 1. Introduction

In the last decade, several techniques have been developed for measuring small forces on single DNA and RNA molecules. In these experiments, one end of a molecule is held at a fixed position, while another end of the molecule is extended at constant force or to a fixed position. These techniques include atomic force microscopy (AFM) [1], optical tweezers [2], and magnetic tweezers [3,4], as shown in Fig. 1. All of these instruments are able to measure the force required to stretch DNA under various conditions. In optical tweezers, one end of a single DNA or RNA molecule is attached to a polystyrene bead in an optical trap, while the other end is held a fixed distance from the trap (Fig. 1A). The resulting force on the bead in the trap is measured over a force range of 0.1 piconewton (pN) to 150 pN. In magnetic tweezers, one end of a single DNA molecule is attached to a magnetic bead, while the other end is attached to a glass surface (Fig. 1B). The magnetic field exerts a constant force, so the resulting extension of the molecule as a function of force can be measured. This method can be used to measure forces well below 1 pN. In atomic force microscopy experiments, DNA molecules are attached to a fixed surface at one

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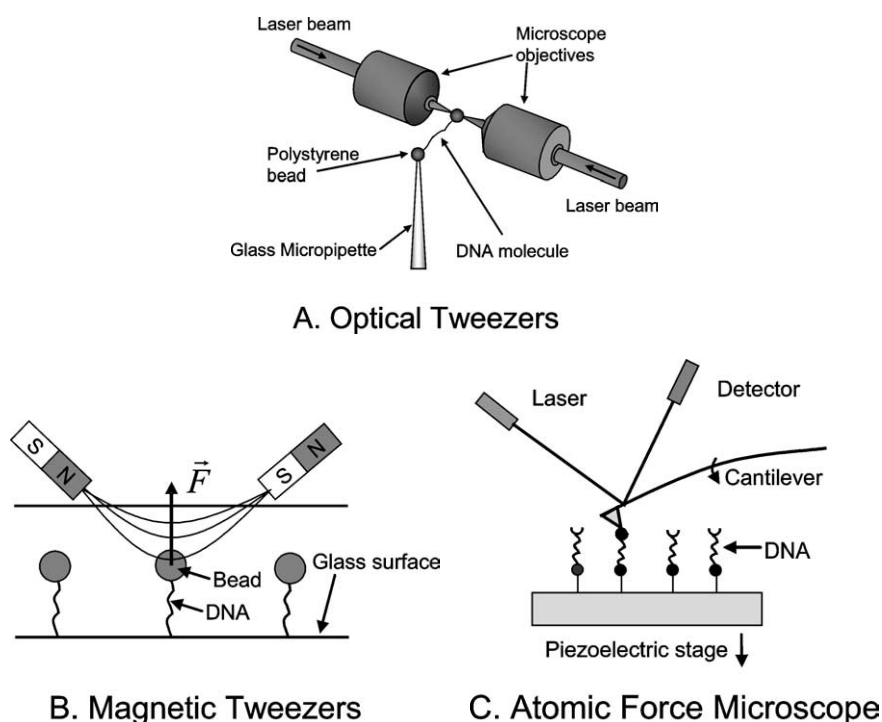


Fig. 1. (A) In an optical tweezers instrument, one or two laser beams are focused to a small spot, creating an optical trap that attracts polystyrene beads. Single DNA molecules are attached at one end to a bead in the trap, while the other end is attached to a movable surface, which in this example is another bead on a glass micropipette. As the DNA molecule is stretched by moving the micropipette, the resulting force on the bead in the trap is measured. (B) In a magnetic tweezers instrument, single DNA molecules are attached at one end to a glass tube, while the other end is attached to a magnetic bead. Magnets located outside the tube generate a magnetic field that exerts a constant force on the magnetic bead. The extension of the DNA molecule as a function of the applied force is then measured. (C) In an atomic force microscopy experiment, single DNA molecules are attached to a surface. The other end of one of these molecules is attached to a cantilever. As the surface is pulled away from the cantilever, the deflection of the cantilever is monitored by measuring the position of a reflected laser beam, which determines the force required to stretch the attached DNA molecule. Figure is taken from [7].

end and a cantilever at the other end (Fig. 1C). As the fixed surface is pulled away from the cantilever, the deflection of the cantilever can be used to determine the force required to stretch the DNA molecule. The resolution of this method is about 5 pN, but it can also be used to measure forces in the nanonewton range. This wide range of techniques has been used to study several regimes of DNA stretching behavior in detail and has also been extended to study DNA–protein and DNA–drug interactions. The use of these force spectroscopy techniques to study macromolecules and proteins has recently been reviewed [5]. The use of DNA and RNA stretching measurements to study DNA– and RNA–protein interactions [6,7] as well as DNA–ligand [7] interactions has also been reviewed.

In Section 2, we will discuss the wide range of experiments that have been done to probe the behavior of DNA at high forces and we will discuss the types of structural transitions that may occur upon stretching. We show that at these high forces a transition occurs in which DNA is melted and its base pairs are broken. These experiments allow us to measure the DNA melting free energy as a function of solution conditions [8,9]. Such free energy measurements can then be used to measure the binding of proteins that alter DNA base-pairing, such as nucleic acid chaperone proteins [10,11] and single-stranded DNA binding proteins [12]. These DNA–protein interaction measurements are discussed in Section 3.

## 2. Force-induced melting of single DNA molecules

### 2.1. Torsionally relaxed DNA

As a single molecule of double-stranded DNA (dsDNA) is stretched beyond its B-form contour length, the force required to stretch the molecule increases dramatically. If one end of the DNA molecule is allowed to rotate freely, at about 65 pN, a cooperative overstretching transition occurs, in which very little additional force is required to stretch the molecule to 1.7 times its contour length, as shown in Fig. 2 [2,13]. To describe this transition, a model of overstretched DNA as a new double-stranded form of DNA, referred to as S-DNA, was proposed [13]. While these models did predict an overstretching transition, the predicted transition was less cooperative and at a higher force than that observed experimentally [14–16]. These S-DNA models were inspired in part by early observations of a stretched form of dsDNA in large DNA bundles [17]. However, in that study the stretched form only appeared upon dehydration of DNA that is within a large fiber bundle. Since those conditions should significantly alter the interactions that cause DNA base pairing and stacking, it is not clear how the transition in that early study can be directly related to the structural transition that occurs when a single DNA molecule is stretched in solution.

Rouzina and Bloomfield [18,19] have proposed an alternative model for DNA overstretching as a force-induced melting process. In this model, the base pairs holding the two DNA strands together break as the DNA molecule unwinds during the transition. This model was shown to be consistent with all available data on the dependence of DNA overstretching on changes in solution conditions such as ionic strength and temperature [20]. It has also been shown that poly(dG·dC)poly(dG·dC) has an overstretching transition about 30 pN higher than poly(dA·dT)poly(dA·dT) [21]. The observation that poly(dA·dT)poly(dA·dT) melts at a force of 35 pN is completely inconsistent with the earlier S-DNA simulation, which showed a B–S transition for poly(dA·dT)poly(dA·dT) of 140 pN [13]. In contrast, the difference in the overstretching force of poly(dG·dC)poly(dG·dC) relative to poly(dA·dT)poly(dA·dT)

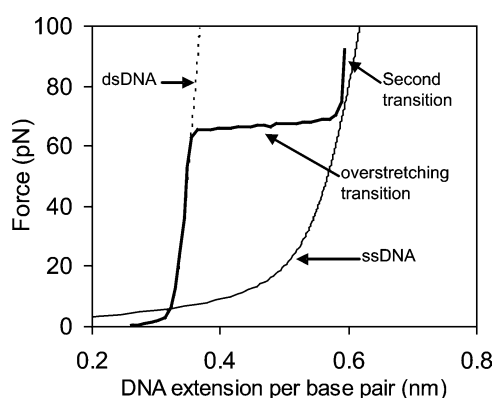


Fig. 2. Typical force–extension curve for stretching dsDNA at pH 7.5 and 500 mM ionic strength (thick solid line). Theoretical force–extension curves for dsDNA (dashed line) and ssDNA (thin solid line) are also shown. The dashed line on the left is a fit of the stretching data to the extensible wormlike chain (WLC) model for the elasticity of a stiff polymer [44]. This curve shows that a much greater force would be required to stretch the DNA beyond the B-form contour length if it remained in its standard helical form. The thin solid line on the right is a fit of data from the stretching of single-stranded DNA (ssDNA) [2] to the extensible freely jointed chain model (FJC) [3]. This model describes the elasticity of a flexible polymer. The transition at approximately constant force near 65 pN is known as the overstretching transition. An additional transition occurs at higher forces before the dsDNA stretching curve exactly matches that of ssDNA.

is consistent with the difference in melting temperature between these molecules. In addition, the authors observed a noncooperative transition at forces higher than the overstretching force as the molecular extension approached that of single-stranded DNA (ssDNA) (see Fig. 2, “second transition”). In a later work [22], they showed that this second transition force depended on the rate at which the dsDNA was stretched. In the force-induced melting theory, the overstretching transition is an equilibrium melting transition, while the second transition at higher force is a non-equilibrium strand separation transition, during which the last base pairs holding the two strands together are irreversibly broken. A rate-dependent force is expected when single bonds are irreversibly broken [23]. In this model, the second transition will occur before the DNA molecule is completely melted, so the average twist of the DNA is expected to be non-zero at the end of the first transition.

To test the force-induced melting model, Williams et al. measured DNA overstretching as a function of pH [8]. Since extremely high and low pH lower the melting temperature of dsDNA, the overstretching force should also decrease if melting occurs during the transition. This decrease in the overstretching force was demonstrated, and the fitted value of the change in entropy of DNA upon melting was in agreement with calorimetric measurements of this parameter at room temperature. As a further test, Williams et al. also measured the temperature dependence of DNA overstretching [9]. While their data were consistent with earlier measurements using AFM [22], the high resolution data obtained using optical tweezers allowed them to directly calculate the helix-coil transition free energy as a function of temperature from the force–extension curves. The resulting parameters describing this temperature dependence, which are the heat capacity of DNA upon melting as well as the entropy upon melting at the melting temperature, were in very good agreement with independent calorimetric measurements of these parameters. Finally, measurements of the monovalent salt dependence of DNA overstretching showed that the DNA strands must remain close together during the transition [24]. The salt dependence of the DNA overstretching transition is consistent with both the S-DNA and force-induced melting model. However, the dependence of the hysteresis observed in these experiments on solution conditions supports the force-induced melting model. This work has recently been reviewed [20]. The force-induced melting model also predicts that DNA binding ligands that alter DNA thermal melting should correspondingly alter DNA force-induced melting. A test of this prediction was carried out by Krautbauer et al., which fully confirms the predicted behavior [25].

## 2.2. Torsionally constrained DNA

dsDNA that is not allowed to rotate freely when stretched does not exhibit an overstretching transition at 65 pN. Instead, a much less cooperative transition at a force of 110 pN is observed [26]. It has been shown that, after unwinding the DNA, the stretching curve exhibits two transitions, one at 50 pN and another at 110 pN and as the amount of DNA unwinding is increased, more of the transition occurs at 50 pN [26]. Overwinding the DNA results in an additional transition at 25 pN, which is attributed to the removal of DNA supercoiling [26]. The currently accepted model is one in which the data are interpreted as transitions between five separate forms of DNA [27]. However, since it is known that underwound DNA is locally denatured even at low forces [28], it seems likely that DNA denaturation occurs during the high force transition as well, but this will require further study. In particular, a detailed study of the dependence of these transitions on solution conditions would help to explain the effect of torsional strain on DNA overstretching. Since torsional strain can build up under many physiological conditions [29], this is an important problem to solve.

The measurements in [26] have been suggested to indicate that the S-DNA created at the end of the transition has a helical twist of 38 bp/turn because this is the amount of helicity remaining in the DNA

molecule when the partially untwisted DNA transition matches the torsionally unconstrained DNA transition at its maximum extension. Similarly, Bryant et al. recently measured the amount of DNA untwisting that occurs as DNA is overstretched [30]. They found that at the end of the transition the average twist of the DNA was 33 bp/turn. Thus, while the studies of DNA untwisting during overstretching do not completely agree, they both show that overstretched DNA retains some overall twist at the end of the transition. However, these experiments do not probe the interactions between the two strands, so they cannot be used to distinguish a final state containing both denatured DNA and B-form DNA from a final state with a specific 'S-DNA' structure. Previous studies have shown that, at very high forces, ssDNA can be stretched to lengths greater than 0.75 nm per base pair [31]. At the end of the overstretching transition, the length of the DNA molecule is 0.58 nm per base pair. Thus, even two non-interacting strands that were held together at a few locations would not completely unwind when stretched to 0.58 nm/bp.

### 2.3. Conclusions: Force-induced melting of DNA

Single molecule force spectroscopy measurements of the DNA overstretching transition have been used to obtain measurements of the transition free energy as a function of solution conditions [20] as well as torque and twist measurements that reveal the average helicity of the molecule after the transition [26,30]. The transition free energy and its dependence on solution conditions match that expected for DNA melting [20]. Therefore, while the exact structure of overstretched DNA is not known [5,30,32], overstretching in the absence of torsional strain must involve DNA denaturation and unwinding. This conclusion is strongly supported by our work, discussed below, on the effects of the ssDNA binding protein T4 gene 32 (gp32), which binds strongly to ssDNA and lowers the DNA overstretching transition force [12].

## 3. Single DNA molecule denaturation by T4 gene 32 protein

Single-stranded DNA binding proteins (SSBs) bind selectively to ssDNA and play important roles in DNA replication, recombination and repair [33]. Although the details of how SSBs function in these processes are not fully understood, they likely interact with ssDNA and prevent the formation of secondary structure [34]. Because single-stranded DNA binding proteins are found in viruses and all three domains of life [35] and are essential for virtually all DNA functions [36], it is important to understand how helix-destabilization by SSBs is regulated. Measurement of the helix-destabilizing capabilities of the well-studied SSB T4 gene 32 protein (gp32) also provides a good test of the model of DNA overstretching as force-induced melting.

Truncation of the 301-residue T4 gene 32 protein (Fig. 3) alters the nucleic acid- and protein-interactive properties of the protein. The first 21 amino acid residues (N-domain) are responsible for binding cooperativity, the core domain (residues 22–253) contains the nucleic acid binding surface [37, 38], and the acidic C-domain (254–301) is involved in binding other T4 replication, repair, and recombination proteins. When the C-domain is removed, with or without the retention of the N-domain, the resulting truncates, \*I and \*III, respectively, possess helix-destabilizing activity as measured in thermal melting experiments [39,40]. \*I, which retains binding cooperativity, lowers the  $T_m$  of dsDNA by about 50–55°C, consistent with the thermodynamic prediction based on its preferential binding to ssDNA [39, 40]. In contrast, full length gp32 has not been observed to lower the thermal melting temperature of natural dsDNA [34,41,42].

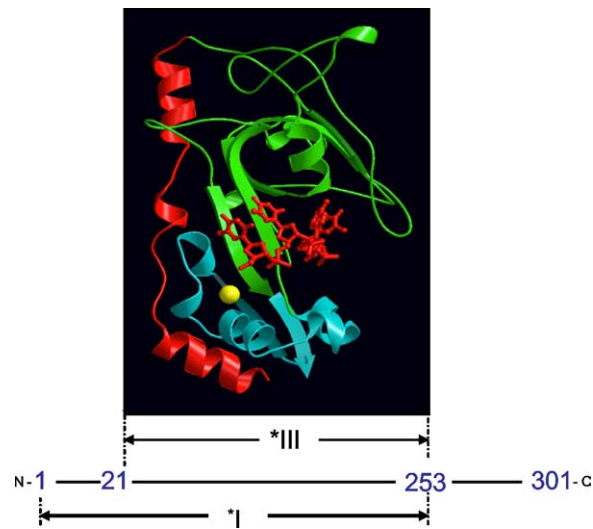


Fig. 3. Proteolytic fragments of gene 32 protein. \*I is obtained by trypsin cleavage of full length gp32 at residue 253, while \*III results from cleavage at residues 21 and 253. A MOLSCRIPT [45] representation of a \*III-oligonucleotide complex is shown at its location within the protein sequence. The protein is pictured in ribbon mode, with the major lobe green, the minor (Zn-containing) lobe blue, and the residue 198–239 flap red. The bound oligonucleotide, in sticks mode, is red, and the coordinated  $Zn^{2+}$ , in space-filling mode, is yellow. The position of the oligodeoxynucleotide, pTTAT, is approximate; it was modeled by Shamoo et al. to maximally overlap excess electron density in the trough [37]. The Protein Data Bank entry for core domain (without the oligonucleotide) is 1gpc.pdb.

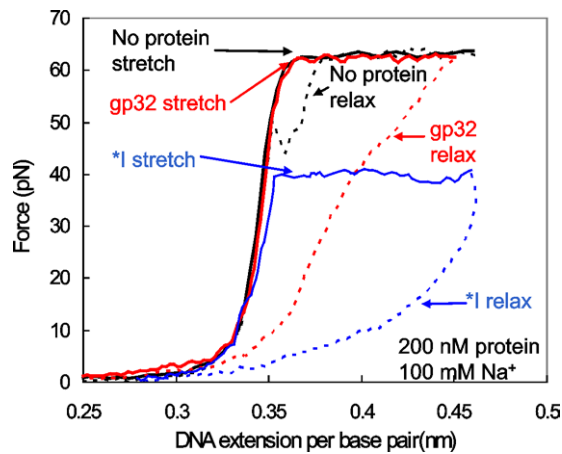


Fig. 4. Stretching (solid line) and relaxation (dashed line) curves for  $\lambda$ -DNA in 10 mM Hepes pH 7.5, 100 mM  $[Na^+]$  (95 mM NaCl and 5 mM NaOH) in the absence of protein (black) and in the presence of 200 nM 32 protein (red) and \*I (blue).

To investigate the helix-destabilization capabilities of gp32 and its proteolytic fragments \*I and \*III, we measured the force–extension curve of  $\lambda$ -DNA over a range of salt and protein concentrations [12]. The results of our measurements in 100 mM  $[Na^+]$  and 200 nM protein are shown in Figs 4 and 5. Within the one minute duration of the experiment, gp32 does not appear to have any effect on the DNA overstretching force, in contrast to \*I, which significantly lowers the transition force. These results suggest that \*I destabilizes DNA under these conditions, while gp32 does not, consistent with the results

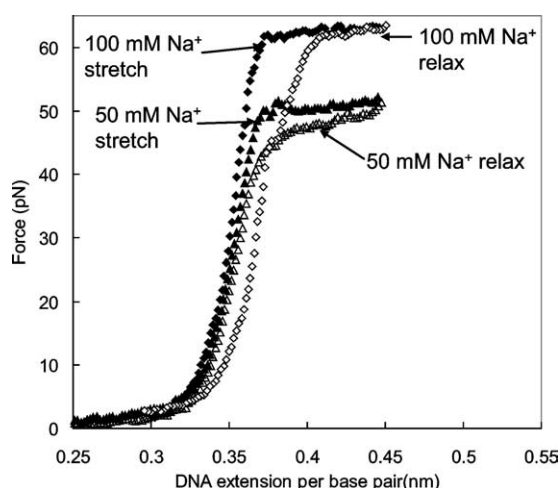


Fig. 5. Stretching (filled symbols) and relaxation (open symbols) curves for  $\lambda$ -DNA in the presence of 200 nM \*III in 10 mM Hepes pH 7.5, 50 mM  $[\text{Na}^+]$  (diamonds) and 100 mM  $[\text{Na}^+]$  (triangles). In both cases, stretching in the presence of \*III shows little hysteresis.

of thermal melting experiments [40]. When the salt concentration is reduced to 50 mM, gp32 also lowers the transition force (data not shown) [12]. When the DNA is relaxed, the relaxation curves for both \*I and gp32 do not match the stretching curves at any point during the overstretching transition. Thus, these proteins bind to exposed regions of ssDNA created when the DNA is stretched, but \*I and gp32 do not dissociate on the one minute time scale of the relaxation experiment [43].

The results shown in Fig. 4 strongly support the idea that overstretched DNA is denatured. gp32 binds very weakly to dsDNA [41] and would not be expected to bind strongly to a base-paired form of DNA due to the larger size of dsDNA. Clearly upon relaxation the protein is bound to ssDNA. The amount of ssDNA created upon stretching is directly determined by the maximum extension to which the DNA molecule is overstretched. The results also show that \*I binds more strongly to ssDNA than gp32, due to the absence of the acidic C-terminus in \*I. Figure 4 also shows that the overstretching transition in the absence of protein is reversible, so those results can be used to obtain the free energy of DNA melting. However, the transition in the presence of gp32 and \*I is not reversible, so free energy measurements cannot be obtained directly from these experiments. In contrast, \*III, which lacks cooperative binding, lowers the transition force but does not induce hysteretic behavior (Fig. 5). Fortunately, free energy measurements can be obtained in the presence of \*I and gp32 by overstretching DNA to a fixed position in the presence of these proteins and measuring the resulting force as a function of time. The force reaches equilibrium after several minutes. The equilibrium force can then be used to calculate an equilibrium melting free energy in the presence of the protein [12].

#### 4. Conclusions

We have described how single molecule force spectroscopy can be used to induce a structural transition in dsDNA known as the DNA overstretching transition. We have presented a model that describes this transition as a force-induced melting transition, or a structural transition from dsDNA to denatured ssDNA. This model makes several quantitative prediction for the transition's dependence on solution conditions, including temperature, pH, salt concentration, and ligand binding. As a further test, we

examined the dependence of the transition force on the presence of a protein (gp32) that binds preferentially to ssDNA. We find that ssDNA binding proteins lower the transition force as predicted by the force-induced melting model and that cooperatively bound protein remains bound to ssDNA as the molecule is relaxed. The results show that single molecule force spectroscopy is a powerful technique for measuring the capability of DNA binding ligands to alter DNA stability. In future work, we will show that this method can be used to measure protein–DNA binding kinetics as well as thermodynamics (K. Pant, I. Rouzina, R.L. Karpel and M.C. Williams, submitted for publication).

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